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Multiple on-line screening and identification methods for hydroxyl radical scavengers in Yudanshen

Yu-Qing Wang*, Guo Zhuang, Shu-Jiao Li

Scientific Research Center, Nanyang Medical College, Nanyang 473061, China

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ABSTRACT

Yudanshen, the genuine medicinal materials of Danshen (Salvia miltiorrhiza), is a well-known traditional Chinese medicine (TCM) used to treat cardiovascular and cerebrovascular diseases. Although its pharmacological and antioxidative activities have been well-documented, there is little research on the hydroxyl radical (•OH) scavenging capacity of Yudanshen. In this study, we established multiple on-line high-performance liquid chromatography- chemiluminescence detector-diode-quadrupole-time of flight mass spectrometry (HPLC-CL-DAD-Q-TOF/MS) methods to rapidly screen and identify the •OH scavengers in Yudanshen simultaneously. The chromatographic and potency fingerprints revealed seventeen peaks that showed the inhibition of •OH. Fourteen of them were identified as danshensu, protocatechuic aldehyde, caffeic acid, ferulic acid, salvianolic acid F, salvianolic acid H/L, salvianolic acid G, salvianolic acid D, salvianolic acid E, rosmarinic acid, salvianolic acid B, isosalvianolic acid B, salvianolic acid A, and salvianolic acid C. This study explores the •OH scavenging activities of Yudanshen, and provides novel and powerful multiple on-line methods in the field of TCM for rapid screening and identification of •OH scavengers.

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1. Introduction

In recent years, reactive oxygen radicals have been implicated in the cause of cancer and other diseases such as stroke, diabetes, cardiovascular diseases, as well as in aging processes [1]. Among these radicals, the hydroxyl radical (•OH) is the most reactive. When it is present in excess or when the cellular antioxidant defense is impaired, the•OH severely damage the neighboring biomolecules in the body, such as protein and DNA, resulting in mutagenesis, carcinogenesis, and cytotoxicity [2,3]. In humans, this oxidative DNA damage by •OH has been proposed as the primary cause of cell-death under oxidative stress conditions [4]. Any compound, natural or synthetic, with radical scavenging properties, can contribute toward the partial or total alleviation of this oxidative damage. Removing •OH is one of the most effective defenses of a living system against diseases, and thus, the •OH scavengers have recently attracted increasing attention [5,6].

Compared to the synthetic compounds, naturally-existing free radical scavengers from traditional Chinese medicine (TCM) have been shown to be less toxic and more effective [7,8]. Danshen (Salvia miltiorrhiza), a well-known TCM, has been used to treat cardiovascular and cerebrovascular diseases, hepatitis, pneumonia, chronic nephritis, and menstrual disorders since ancient times. It is particularly useful for patients with coronary heart disease (CHD), and has been developed into more than thirty pharmaceutical dosage forms [9,10]. Yudanshen, the danshen grown in Fangcheng (county of Nanyang, located in Henan province of China), was recognized as genuine medicinal materials with special characteristics for its high quality and therapeutic effects. Although the antioxidant properties of Danshen have been extensively studied [11,12], there is little information about the potential •OH scavengers found in Yudanshen. Herein we explore the corresponding relationship between chemical composition of Yudanshen and its •OH scavenging activities.

The hydroxyl radical, as the most reactive and dangerous free radical, is very difficult to detect, and existing detection methods cannot be used to screen •OH scavengers in the complex matrixes. In light of the aforementioned facts, we established new multiple on-line high performance liquid chromatography-chemiluminescence detector-diode-quadrupole-time of flight mass spectrometry (HPLC-CL-DAD-Q-TOF/MS) methods to rapidly screen and identify the •OH scavengers in Yudanshen simultaneously. The •OH producing system, which was

* Corresponding author.
E-mail address: wyq2013@163.com (Y.-Q. Wang).

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designed based on the Fenton reaction, was also optimized for online detection.

Proportional valve shunt was used to achieve the synchronization between the online mass spectrometry detection and the active detection system. The chromatographic and potency fingerprints revealed the active components, correlated with the •OH scavenging actions. This research explores the •OH scavenging activities of Yudanshen, and the multiple on-line HPLC coupled with chemiluminescence (CL) detection and Q-TOF/MS methods provide a powerful tool in the field of TCM for rapid identification of •OH scavengers.

2. Materials and methods

2.1. Plant materials

The dried Yudanshen (Salvia miltiorrhizae) were supplied by Yudanshen Planting Base of Good Agricultural Practice (GAP) certification in Fangcheng county of Nanyang (Henan Province, China). Voucher specimens were deposited in the laboratory of the Scientific Research Center of Nanyang Medical College (Henan Province, China).

2.2. Chemicals and reagents

Chromatographic reagent grade acetonitrile (Thermo Fisher Scientific Co. Ltd., Shanghai, China) and analytical reagent grade formic acid (Nanjing Chemical Plant, Nanjing, Jiangsu, China) were used for the preparation of mobile phase. Standards of danshensu, protocatechuic aldehyde, caffeic acid, ferulic acid, salvianolic acid D, salvianolic acid E, rosmarinic acid, salvianolic acid B, salvianolic acid A, and salvianolic acid C were purchased from the Chinese food and drug inspection institute (Beijing, China). Deionized water was prepared by Millipore water purification system (MilliporeSigma, Bedford, MA, USA). Ascorbic acid, 1,10-phenanthroline, Na_2HPO_4 and NaH_2PO_4 were purchased from Sigma company (St. Louis, MO, USA).

Hydrogen peroxide (30% H_2O_2 in water), Na_2CO_3, $NaHCO_3$ and $CuSO_4{\cdot}5H_2O$ were purchased from Shanghai Chemical Reagent Corporation (Shanghai, China). All chemicals were of analytical reagent grade.

The scavenging activities of Yudanshen were determined by using freshly prepared solutions as described below. Solution I was 1.00 mmol/L $CuSO_4{\cdot}5H_2O$; Solution II was 2.00 mmol/L ascorbic acid dissolved in PBS buffer (pH 6.20, 2.00 M); Solution III was 2% H_2O_2; Solution IV was prepared by first dissolving the accurately weighed 1,10-phenanthroline in Na_2CO_3-$NaHCO_3$ buffer (pH 9.60, 2.00 M), and diluting to 2.00 mmol/L. All the solutions were filtered through a 0.45 μm membrane filter prior to on-line activity assay.

2.3. Sample and standard solution preparation

The dried powder of Yudanshen (0.3 g) was passed through a sieve (100 mesh, 0.15 mm) and was soaked in 10 mL methanol (70%, v/v), followed by sonicating at room temperature for 60 min. The resulting mixture were centrifugated at 3000 rpm for 10 min, and the supernatant was collected and filtered through 0.22 μm Econofilter (Agilent Technologies, Santa Clara, CA, USA) prior to chromatographic analysis.

The reference compounds obtained from NICPBP were accurately weighed and dissolved in methanol (70%, v/v) as stock solutions. All samples prepared from the plant materials and reference compounds were stored at 4 °C until use.

2.4. On-line HPLC-CL-DAD-Q-TOF/MS instrumentation

The on-line HPLC-CL-DAD-Q-TOF/MS apparatus consisted of an Agilent HPLC system (Agilent Technologies, Santa Clara, CA, USA), Agilent reagent pump, proportioner, mixing tee, T-junction, reaction coil (15 m × 0.25 mm i.d., PEEK tubing), a diode array detector (DAD), a chemiluminescence (CL) detector and a Q-TOF mass spectrometry detector. The injection volume was set to 15 μL. Briefly, injected sample was first passed through the HPLC system, and separated by the column, then arrived at a proportioner. The optimal split ratio between DAD and the post column CL detection is 2:8. HPLC eluates (20%) was detected by DAD and then entered mass spectrometry detector. The other 80% of the eluates entered into reaction coil in order to be mixed with CL reagents. Then the •OH scavenging activities of analytes were measured by CL detector. The on-line instrumentation was shown in Fig. 1.

2.5. HPLC-DAD analysis

Analysis of the compounds in Yudanshen was performed on Agilent 1260 HPLC system with a diode array detector (DAD). Samples (15 μL) were separated on an Agilent Zorbax SB C-18 column (250 mm × 4.6 mm i.d., 5 μm particle size) with a gradient elution system. The mobile phase consisted of two solvents: solvent A was a mixture of water/formic acid (99.9/0.1, v/v), and solvent B was 100% acetonitrile.

Gradient elution was performed at 30 °C with a 1.0 mL/min flow rate as follows: 0–22 min, 95.0%-78.4% A; 22–40 min, 78.4%–70.0% A; 40–50 min, 70.0%-60.0% A; 50–55 min, 60.0%-30.0% A; 55–60 min, 30.0%-95.0% A. Spectral data from all peaks were collected in the range of 200–900 nm, and the UV–vis chromatogram was recorded at 280 nm.

2.6. HPLC-CL analysis

Feton reaction was used as the basis for designing an on-line HPLC-CL-DAD detection. Hydroxyl radicals were generated by reaction of Cu(II) with H_2O_2 in the presence of ascorbic acid as acidic medium. When 1,10-phenanthroline, which can trap hydroxyl radicals, is added to the system, CL emission was produced. When the analytes extracted from Yudanshen were added into the reaction system, the •OH radicals were scavenged, and inhibited of the CL emission.

The CL solutions I–IV were separately stored in liquid storage bottles, then injected and delivered individually by multichannel reagent pump (Agilent Technologies, USA). Thereinto, solution I and solution II were delivered by the same pump with a 0.5 mL/min flow rate, solution III and Solution IV were delivered by another identical pump with a 1.0 mL/min flow rate. Solution I and solution II were mixed firstly, and then mixed with the other two solutions in the mixing tee. The mixture of HPLC eluates and CL reagents in the reaction coil (15 m × 0.25 mm i.d. PEEK tubing) were maintained at 30 °C with an Agilent temperature control module (Agilent Technologies, USA), and the chemiluminescence properties were measured by BPCL system (Academia Sinica Biophysics Institute, Beijing, China).

The •OH scavenging properties were evaluated by the scavenging potency calculated as follows:

$$\text{Scavengingpotency} = CL_0 - CL_s/H_s - H_0$$

where CL_0 was the baseline intensity of CL (without sample) and CL_s was the CL intensity inhibited by the compounds present in the extracts; H_0 was the baseline height at 280 nm, H_s was the peak height at 280 nm of every compound present in the extracts.

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